[CANCER RESEARCH 50, 6817–6822, November 1, 1990]

Metabolism of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone in Mouse Lung Microsomes and Its Inhibition by Isothiocyanates[1]

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ABSTRACT

The tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces lung tumors in rats, mice, and hamsters, and metabolic activation is required for the carcinogenicity. 2-Phenethyl isothiocyanate (PEITC), whose precursor gluconasturtiin (a glucosinolate) occurs in cruciferous vegetables, has been found to inhibit carcinogenesis by NNK. The purpose of the study was to investigate the enzymes involved in the metabolism of NNK in lung microsomes and to elucidate the mechanisms of inhibition of NNK metabolism by isothiocyanates. NNK metabolism in lung microsomes (isolated from female A/J mice) resulted in the formation of formaldehyde, 4-hydroxy-1-(3-pyridyl)-1-butanone (keto alcohol), 4-oxo-4-(3-pyridyl)butyric acid (keto acid), 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, displaying apparent K_m values of 5.6, 5.6, 9.2, 4.7, and 2540 μM, respectively. Higher K_m values in the formation of formaldehyde and keto alcohol were also observed. When cytochrome P-450 inhibitors [2-(diethylamino)ethyl 2,2-diphenylpentenoate] hydrochloride (100 μM), carbon monoxide (90%), and 9-hydroxyellipticine (10 μM) were used, NNK metabolism was inhibited by each 70, 100, and 30%, respectively. Methimazole (1 mM), an inhibitor of the flavin-dependent monooxygenase, inhibited the formation of 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol by 20%, but had no effect on the formation of keto alcohol. Inhibitory antibodies against cytochromes P-450IIB1 and -2, P-450IA1, and P-450IA2 inhibited the formation of keto alcohol by 25, 15, and 0%, respectively. Administration of PEITC at doses of 5 and 25 μmol/mouse 2 h before sacrifice produced a 40 and 70% decrease in microsomal NNK metabolism, respectively. PEITC and 3-phenylpropyl isothiocyanate exhibited a mixed type of inhibition, and the competitive component of inhibition had apparent K_i values of 90 and 30 nM, respectively. Preincubation of PEITC in the presence of a NADPH-generating system did not result in a further decrease in the formation of NNK metabolites, indicating that the metabolism of PEITC was not required for the inhibition. When a series of isothiocyanates with varying alkyl chain length (phenyl isothiocyanate, benzyl isothiocyanate, PEITC, 3-phenylpropyl isothiocyanate, and 4-phenylbutyl isothiocyanate) were used, the potency of the inhibition increased with the increase in chain length. The results suggest that cytochromes P-450 are involved in the metabolism of NNK and that the inhibitory action of isothiocyanates is due to competitive inhibition and an inactivation of the enzyme by these compounds.

INTRODUCTION

NNK[3] is a potent tobacco-specific carcinogen formed from the nitrosation of nicotine during tobacco processing and cigarette smoking (1–3). It has been found to induce tumors in the nasal cavity, lung, and liver of rats (4); the skin and lung of mice (5, 6); as well as the trachea, nasal cavity, and lung of hamsters (7). Metabolic activation of NNK is believed to involve the α-hydroxylation of either the methylene carbon leading to the formation of keto aldehyde and methyldiazohydroxide or the methyl carbon leading to the formation of formaldehyde and 4-(3-pyridyl)-4-oxobutyldiazohydroxide. The latter compound is a hypothetical precursor of the metabolite keto alcohol (Fig. 1) (8, 9). Generation of the diazohydroxides, which can cause DNA alkylation, may be responsible for the potent carcinogenicity of NNK. However, the enzymes that are responsible for the activation process are not well established. Devereux *et al.* (10) reported that a homologue of rabbit cytochrome P-4502 (cytochrome P-450IIB4 or P-450b[4]) and a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin inducible cytochrome P-450 isozyme (P450IA1 or P-450c) may be involved in the activation of NNK in Clara cells from rats.

Isothiocyanates are compounds that occur as glucosinolates in a variety of cruciferous vegetables such as cabbage and Brussels sprouts (11, 12). When the raw vegetables are wet and crushed, the glucosinolates are hydrolyzed by the plant enzyme myrosinase releasing glucose and forming isothiocyanates as products (11). BITC and PEITC are two naturally occurring isothiocyanates. PEITC at a daily dosage of 5 and 25 μmol/mouse for 4 consecutive d has been found to inhibit NNK-induced DNA adduct formation and tumorigenicity in mice (13). Administration of 1 mmol PEITC/kg body weight to rats significantly inhibited hepatic *N*-nitrosodimethylamine demethylase activity (14). Recently, the inhibitory potency of a series of isothiocyanates on lung tumorigenesis has been found to be related to the alkyl chain length. 2-Phenethyl isothiocyanate, PPITC, and PBITC significantly inhibited lung tumorigenesis induced by NNK, whereas the shorter-chain isothiocyanates PITC and BITC were ineffective (15). The present study was undertaken to characterize the enzymes responsible for the metabolism of NNK in lung microsomes and to elucidate the mechanisms of inhibition by isothiocyanates.

MATERIALS AND METHODS

Chemicals. Unlabeled NNK, [5-^{3}H]NNK (2.20 Ci/mmol; purity >98%), and [^{3}H-*methyl*]NNK (1.06 Ci/mmol; purity >95%) were purchased from Chemsyn Science Laboratories (Lenexa, KS). PEITC, PITC, and BITC were purchased from Aldrich Chemical Company (Milwaukee, WI). PPITC was purchased from Fairfield Chemical Company (Blythewood, SC). PBITC and NNK metabolite standards were synthesized as described previously (8, 15). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP$^+$, EDTA, magnesium chloride, methimazole, and barium hydroxide were purchased from Sigma Chemical Company (St. Louis, MO). Zinc sulfate and Scintiverse LC

Received 4/6/90; accepted 8/1/90.

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[1] This study was supported by NIH Grants CA-46535 and CA-37037, and a fellowship from the New Jersey State Commission on Cancer Research.

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[3] The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; keto alcohol, 4-hydroxy-1-(3-pyridyl)-1-butanone; keto acid, 4-oxo-4-(3-pyridyl)butyric acid; BITC, benzyl isothiocyanate; PEITC, 2-phenethyl isothiocyanate; PPITC, 3-phenylpropyl isothiocyanate; PBITC, 4-phenylbutyl isothiocyanate; PITC, phenyl isothiocyanate; NNK-*N*-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone; HPLC, high-performance liquid chromatography; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; V_{max}, maximal velocity.

[4] The following systematic and trival names of cytochromes P-450 are used: cytochrome P-450IIB1, P-450b; cytochrome P-450IIB1 and -2, P-450b and -e; cytochrome P-450IIB4, rabbit $P450_{LM2}$; cytochrome P-450IA1, P-450c; cytochrome P-450IA2, P-450d.

scintillation cocktail were obtained from Fisher Scientific Company (Fair Lawn, NJ). 9-Hydroxyellipticine was a gift from Pierre Lesca (Institut de Toxicologie, Toulouse, France). [2-(Diethylamino)ethyl 2,2-diphenylpentenoate]hydrochloride was a gift from Research Biochemicals (Wayland, MA). Monoclonal antibodies to rat cytochromes P-450IIB1 and -2, and P450IA1, polyclonal antibodies to rat cytochrome P-450IA2, and nonimmune serum were prepared and characterized as described previously (16–18).

Animals. Female A/J mice 5 weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained on an AIN-76A semipurified pelleted diet (ICN Biochemicals, Cleveland, OH) for 1 week. Mice were house 10 per cage in polycarbonate cages on corncob bedding and were maintained in an air-conditioned room with a 12-h light and dark cycle. For the experiment on the effect of PEITC *in vivo*, mice were given doses of 5 or 25 μmol/mouse in 100 μl of corn oil intragastrically 2 h prior to sacrifice.

NNK Metabolism Catalyzed by Lung Microsomes. Lung microsomes were prepared and isolated as described previously (13) and stored at −70°C. The cytochrome P-450 content and protein concentration were determined using previously described methods (19, 20). Unless otherwise stated, the incubation mixture consisted of 100 mM sodium phosphate, pH 7.4, 5 mM glucose-6-phosphate, 1.52 units of glucose-6-phosphate dehydrogenase, 1 mM $NADP^+$, 1 mM EDTA, 3 mM $MgCl_2$, 10 μM NNK (1 μCi of [5-^{3}H]NNK and 1 μCi of [^{3}H-*methyl*]NNK), and 0.1 mg of microsomal protein in 400 μl. The reaction mixture was incubated at 37°C for 30 min and terminated by the addition of 100 μl each of 25% zinc sulfate and saturated barium hydroxide. The sample was centrifuged and filtered through a 0.45 μm ACRO LC3A filter disc (Fisher Scientific Company), and 200 μl were coinjected with 5 μl of NNK metabolite standards onto a reverse-phase HPLC system. The HPLC system consisted of a Waters automated gradient controller, two Waters 6000A pumps, Waters 710B WISP auto-injector, Waters 440 UV detector, and a C_{18} μBondapak column (3.9 x 300 mm; Waters, Milford, MA). It was eluted with a linear gradient of 95% A (0.02 M Tris-HCl buffer, pH 7.0) and 5% B (methanol) to 65% A and 35% B over a 50-min period at a flow rate of 1 ml/min. The radioactive peaks were quantified by a Radiomatic Flo-One/Beta radioactive flow detector. Isothiocyanates when used *in vitro* were dissolved in 95% ethanol. Ethanol had no effect on NNK metabolism when used at 1% of the incubation volume.

RESULTS

Identification of Metabolites. Metabolism of NNK in mouse lung microsomes led to the formation of formaldehyde, keto acid, NNK-*N*-oxide, keto alcohol, and NNal (Fig. 2). The

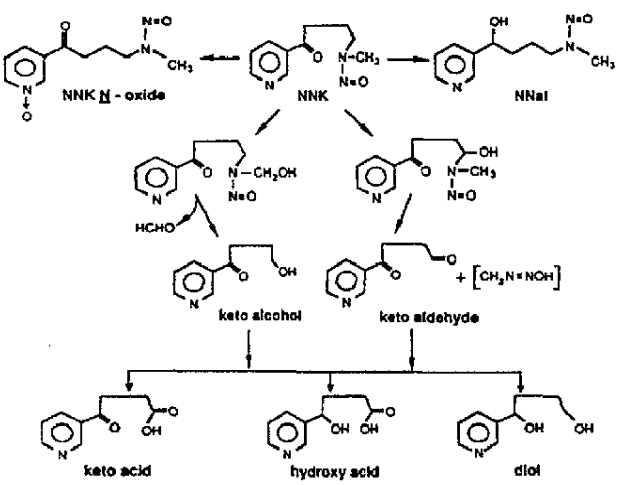

Fig. 1. Metabolism of NNK. Modified from Hecht *et al.* (8).

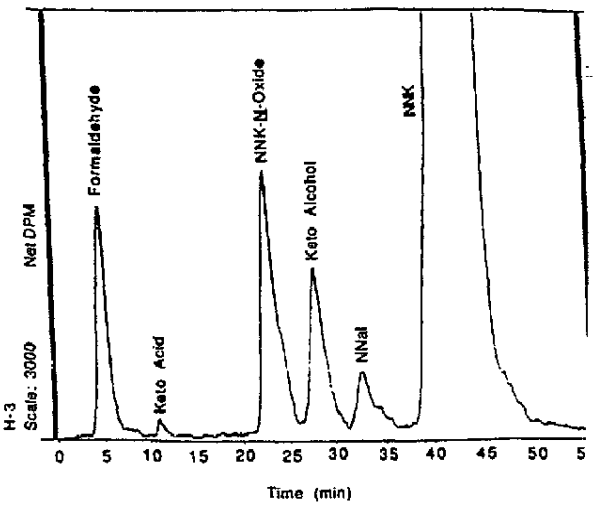

Fig. 2. Radioactive chromatogram of NNK metabolism in mouse lung micr somes. *In vitro* incubation mixture consisted of microsomal protein (0.1 mg); μM NNK (1 μCi of [5-^{3}H]NNK and 1 μCi of [^{3}H-*methyl*]NNK); a NADPH generating system; 100 mM sodium phosphate buffer, pH 7.4; 1 mM EDTA; a 3 mM $MgCl_2$ in a total volume of 400 μl. Reactions were carried out for 30 m at 37°C. DPM, disintegrations per minute.

identities of the NNK metabolites were determined by coelutic with authentic standards. Formaldehyde was identified by co lecting the peak and coinjecting it with a known amount of [^{3}H formaldehyde. Keto acid was further identified by collecting th peak and subjecting the sample to HPLC analysis using mobil phase buffers at pH 4, 4.5, 5, 6, and 7 as described by Carmel and Hecht (21). As the pH decreased, the retention time of th keto acid standard and collected fraction increased (data n shown).

The formation of the NNK metabolites was found to be linea with time up to 60 min (data not shown). When a 10 μM NN concentration was used, approximately 10% of the NNK wa metabolized by the lung microsomes in a 30-min incubatio Two major metabolites, formaldehyde and keto alcohol, we formed in approximately a 1:1 ratio. By using equal amoun of NNK molecules labeled at the α-methyl and pyridyl-5 pos tions, both metabolites were analyzed in the same assay. Sin both metabolites are expected to be formed as a result of th oxidation of the α-methyl group (Fig. 1), the result was co sistent with the theoretical ratio. A predicted NNK metabolit keto aldehyde (8), was not observed under the assay conditior This may be due to the possibility that keto aldehyde w oxidized to keto acid or became bound to microsomal protei

Substrate Dependency of NNK Metabolism. In the substra concentration range of 1 to 20 μM NNK, apparent Michael Menton kinetics were observed in the formation of formald hyde, keto alcohol, NNK-*N*-oxide, and keto acid (Fig. 3*A*). T apparent K_m and V_{max} values are summarized in Table 1. T formation of formaldehyde and keto alcohol had the sar apparent K_m, 5.6 μM, and V_{max}, 57 pmol/min/mg protei consistent with the idea that these two metabolites are form from the same reaction. The formation of keto acid involved least one more oxidation step, and an apparent K_m of 9.2 μ was observed. The K_m for the formation of NNK-*N*-oxide, 4 μM, was not significantly different from the K_m for formalc hyde and keto alcohol. However, the data do not allow us conclude that the same enzyme was involved in the formati

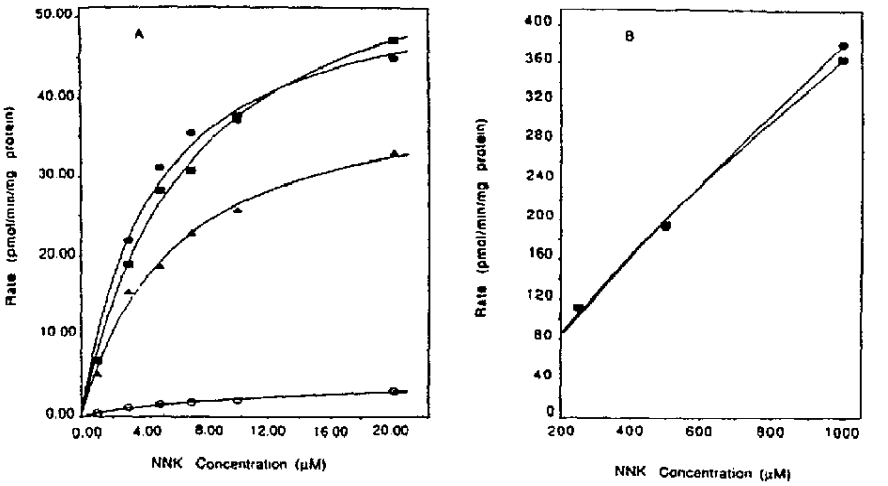

Fig. 3. Substrate dependency of the metabolism of NNK. The incubation mixture consisted of microsomal protein (0.1 mg); a NADPH-generating system; 100 mM sodium phosphate buffer, pH 7.4; 1 mM EDTA; 3 mM $MgCl_2$, and 1–20 μM NNK (A) and 250–1000 μM NNK (B) (1 μCi of [5-^{3}H]NNK and 1 μCi of [^{3}H-*methyl*]NNK) in a total volume of 400 μl. Reactions were carried out for 30 min at 37°C, and the formation of keto alcohol (●), formaldehyde (■), NNK-*N*-oxide (▲), and keto acid (○) were determined. The activity of the formation of NNK metabolite is expressed as pmol/min/mg protein. *Points*, mean of four replications; difference between the replications was less than 10%.

Table 1 *Kinetic parameters for NNK metabolism in mouse lung microsomes*[a,b]

Metabolite	K_m (μM)	V_{max} (pmol/min/mg protein)
Formaldehyde	5.6 ± 0.9[1]	57.2 ± 2.2[4]
Keto alcohol	5.6 ± 0.9[1]	56.0 ± 3.8[4]
Keto acid	9.2 ± 1.0[2]	4.2 ± 0.5[5]
NNK-*N*-oxide	4.7 ± 0.9[1]	54.2 ± 1.3[4]
NNal	2540.7 ± 15.4[3]	1322.3 ± 10.2[6]

[a] Values are the mean ± SD of four replications.
[b] Means with different superscripts in the same column are significantly different ($P < 0.01$) from each other as determined by analysis of variance followed by the Newman-Keuls range test.

Table 2 *Effects of enzyme inhibitors on NNK metabolism*[a,b]

Inhibitor concentration (μM)	Metabolite formation (% inhibition) HCHO	Keto alcohol	Keto acid	NNK-*N*-oxide	NNal
SKF-525A[c]					
50	54 ± 5[1]	58 ± 8[1]	49 ± 2[1]	63 ± 6[1]	37 ± 2
100	70 ± 2[2]	72 ± 5[1]	100[3]	71 ± 2[2]	29 ± 4
200	69 ± 6[2]	71 ± 3[1]	100[3]	67 ± 7[1]	26 ± 2
Carbon monoxide[d]					
50%	61 ± 4[1]	62 ± 5[1]	100[2]	100[2]	0
75%	76 ± 2[2]	72 ± 2[2]	100[2]	100[2]	31 ± 2
90%	100[4]	100[4]	100[2]	100[2]	32 ± 1
9-Hydroxyellipticine					
1	30 ± 9[1]	21 ± 6	30 ± 2	19 ± 3	20 ± 1
5	34 ± 1[1]	31 ± 2[1]	47 ± 2[1]	19 ± 1	13 ± 3
10	32 ± 6[1]	30 ± 1[1]	44 ± 2[1]	22 ± 6	15 ± 1
Methimazole					
100	15 ± 3[1]	3 ± 2	100[2]	7 ± 2	10 ± 3
500	20 ± 3[1]	4 ± 3	100[2]	23 ± 4[1]	31 ± 7
1000	18 ± 0[1]	0	100[2]	20 ± 3[1]	25 ± 4

[a] Microsomal incubation mixtures were preincubated with the inhibitors for 5 min. The reaction were initiated by the addition of substrate. Rates of metabolite formation in control lung microsomes were 35.5, 36.9, 1.8, 26.9, and 14.8 pmol/min/mg protein for HCHO, keto alcohol, keto acid, NNK-*N*-oxide, and NNAl, respectively.
[b] Values are the mean ± SD of three replications. Analysis of variance followed by the Newman-Keuls range test was performed on the original data; datum points with different superscripts in the same column are significantly ($P < 0.05$) different from the control and each other. For clarity only the number of percent inhibition is shown.
[c] SKF-525A, [2-(diethylamino)ethyl 2,2-diphenyl pentenoate]hydrochloride.
[d] A mixture of carbon monoxide and air was bubbled through the microsome-buffer incubation mixture for 3 min. The reaction was initiated by the addition of substrate and a NADPH-generating system.

of these metabolites. The apparent K_m for the formation of NNal was very large (2540 μM), reflecting the fact that carbonyl reductase is responsible for this pathway (1). In addition to the low K_m enzyme form in the oxidation of NNK, the involvement of higher K_m forms of enzymes in the formation of formaldehyde and keto alcohol was also evident (Fig. 3*B*). Saturation kinetics for formaldehyde and keto alcohol formation was not observed in the NNK concentration range of 250 to 1000 μM. The formation of keto acid and NNK-*N*-oxide, however, was not detectable at NNK concentrations higher than 250 μM, possibly due to substrate inhibition.

Effects of Inhibitors. To characterize the enzymes involved in NNK metabolism, various inhibitors were utilized (Table 2). [2-diethylamino)ethyl 2,2-diphenyl pentenoate]hydrochloride, a commonly used cytochrome P-450 inhibitor, completely inhibited keto acid formation, and inhibited formaldehyde, keto alcohol, and NNK-*N*-oxide formation by approximately 70%. At 90%, carbon monoxide completely inhibited the formation of formaldehyde, keto alcohol, keto acid, and NNK-*N*-oxide. The inhibitor 9-hydroxyellipticine, which is specific for cytochrome P-450IA1 and -IA2 (P-450c and d) in rat liver (22), produced a 30–40% inhibition of formaldehyde, keto alcohol, and keto acid, but only approximately 20% inhibition in the formation of NNK-N-oxide. With all of the above inhibitors, NNal was inhibited to a far lesser extent than the other metabolites. Methimazole, an inhibitor of the flavin-dependent monooxygenase (23), completely inhibited keto acid formation and had no effect on keto alcohol formation, whereas the formation of formaldehyde, NNK-*N*-oxide, and NNal was inhibited by 20%.

Inhibitory antibodies against cytochromes P-450IA1 (P-450c), P-450IIB1 and -2 (P-450b and e), and P-450IA2 (P-450d) were used to further investigate the involvement of these or immunochemically related cytochrome P-450 isozymes in NNK metabolism (Table 3). Anti-P-450IA1 produced a 15% inhibition of the formation of keto alcohol. Anti-P450IIB1 and -2, at a concentration that completely inhibited pentoxyresorufin dealkylase (a P-450IIB1 specific reaction), inhibited keto alcohol and keto acid formation by 25% and had no effect on the formation of NNK-*N*-oxide and NNal. Anti-P-450IA2, on the other hand, had no effect on NNK metabolism. The amounts of antibodies were saturating; additional inhibition was not observed with higher quantities of antibodies (data not

Table 3 *Effects of cytochrome P-450 specific antibodies on the formation of metabolites from NNK and pentoxyresorufin*[a,b]

Antibody	(pmol/min/mg protein) Keto alcohol	Keto acid	NNK-*N*-oxide	NNal	Inhibition of resorufin[c]
C7 (control)	34.2 ± 1.1	2.0 ± 0.2	23.1 ± 1.3	14.6 ± 0.5	
Anti-IA1	29.1 ± 0.9[1] (15)[d]	1.62 ± 0.1 (19)	20.6 ± 0.2 (11)	13.4 ± 0.3 (8)	
Anti-IIB1 and -2	25.7 ± 0.8[1] (25)	1.54 ± 0.2 (23)	22.4 ± 0.2 (3)	14.9 ± 1.1 (0)	(100)
Anti-IA2	35.3 ± 1.2 (0)	2.2 ± 0.7 (0)	22.6 ± 0.2 (2)	15.1 ± 0.2 (0)	

[a] Monoclonal antibodies C7 (control), anti-IA1 (C8), and anti-IIB1 and -2 (BE52) were added to lung microsomes at a concentration of 1 mg/nmol cytochrome P-450 (17, 18). Polyclonal antibodies [gG (control) and anti-IA2 [anti-P-450d(-c)] (16) were added at a concentration of 10 mg/nmol cytochrome P-450. The microsomal-antibody mixture was allowed to react for 5 min at room temperature before being used in incubations. The reaction was initiated with the NADPH-generating system and 10 μM NNK (containing 1 μCi of [5-^{3}H]NNK).

[b] Values are the mean ± SD of three replications. Means with superscript[1] are significantly ($P < 0.05$) different from the control as determined by the Student's *t* test.

[c] A product of pentoxyresorufin *O*-dealkylation. The incubations contained 10 μM pentoxyresorufin and 1.5 mg of microsomal protein in a total volume of 2 ml. The rate by control mouse lung microsomes was 5.9 pmol resorufin/min/mg protein.

[d] Numbers in parentheses are the percentage inhibition of metabolite formation compared with control.

shown). To determine whether prostaglandin synthetase was involved in NNK metabolism, arachidonic acid (1 mM) was used in place of the NADPH-generating system. Metabolism of NNK was not observed (data not shown), suggesting that prostaglandin synthetase is not involved. The above results suggest that NNK is metabolized by cytochromes P-450, and that isozymes similar to cytochromes P-450IIB1 and P-450IA1 appear to be involved.

Inhibition of NNK Metabolism by PEITC. A/J mice were given corn oil, 5 μmol of PEITC, or 25 μmol of PEITC intragastrically 2 h prior to sacrifice, and the activity of the lung microsomes in metabolizing NNK was analyzed. After a dose of 5 μmol of PEITC, the rate of the formation of formaldehyde, keto alcohol, and NNal decreased by approximately 30% and NNK-*N*-oxide by 40%. With a dose of 25 μmol of PEITC, formaldehyde, keto alcohol, and NNK-*N*-oxide, formation was decreased by 70–80% (Table 4). When the microsomes of the latter group of mice were used to study the formation of formaldehyde, keto alcohol, and NNK-*N*-oxide, in incubations containing 1–20 μM NNK, the K_m was higher and the V_{max} was lower than those obtained with the control microsomes (data not shown).

When 50 nM PEITC or PPITC was added to reaction mixtures of NNK metabolism assays, the isothiocyanates exhibited a mixed type of inhibition for keto alcohol formation (Fig. 4). A similar mechanism of inhibition by PEITC and PPITC was also observed for the formation of formaldehyde and NNK-*N*-oxide (data not shown). In the formation of both formaldehyde and keto alcohol, there was a competitive component of inhibition with apparent K_i of 90 and 30 nM (calculated from the slope of the double-reciprocal plot) for PEITC and PPITC, respectively. Since PEITC is known to react with amino, histidyl, and cysteinyl groups in protein, the noncompetitive component of the inhibition is probably due to this chemical reactivity.

In order to study the possible inhibition by metabolites of PEITC, the effect of preincubation of microsomes with PEITC in the presence and absence of a NADPH-generating system were studied (Table 5). The addition of 25 and 50 nM PEITC to the incubation produced the expected inhibitory effect. Preincubation of PEITC with microsomes resulted in even lower metabolic activity. However, the presence of the NADPH generating system in the preincubation did not cause a further decrease in the metabolic activity. Similar results were found in an experiment in which control mouse lung microsomes were preincubated for 20 min with PEITC in the presence and absence of the NADPH-generating system and then diluted 20 fold for the NNK metabolism study (data not shown). The results suggest that PEITC directly binds to and inactivates the cytochrome(s) P-450 responsible for NNK metabolism, and metabolic activation of PEITC is not required for the inactivation.

Relative Inhibitory Strengths of Different Isothiocyanates. The effect of various isothiocyanates with different alkyl chain lengths on NNK metabolism is shown in Table 6. PITC at a concentration of 25 and 100 nM had the least effect on NNK metabolism. PBITC was the most effective in inhibiting NNK metabolism. With an increase in alkyl chain length, the inhibitory potency of the isothiocyanates increased.

DISCUSSION

The activation of NNK occurs by way of α-hydroxylation which can result in the methylation and pyridyloxobutylation of DNA (1–3, 9). Microsomes are believed to be responsible for most of the oxidative metabolism of NNK in the cell. In the present work, products of α-hydroxylation, pyridine-*N*-oxidation, and carbonyl reduction were observed. The observed metabolite profile in lung microsomes was similar to that obtained with cultured A/J mouse lung (24). The presence of low and high K_m forms of enzymes for the formation of formaldehyde and keto alcohol were observed. Both of these metabolites were

Table 4 *Effect of PEITC treatment of mice on the formation of metabolites from NNK in lung microsomes*[a,b]

Treatment	(pmol/min/mg protein) HCHO	Keto alcohol	NNK-*N*-oxide	NNal
Control	33.2 ± 0.1[1]	38.6 ± 2.1[1]	27.3 ± 1.2[1]	11.2 ± 1.0[1]
5 μmol PEITC	21.4 ± 2.2[2]	26.6 ± 0.8[2]	15.4 ± 0.1[2]	7.6 ± 0.9[2]
25 μmol PEITC	10.9 ± 1.1[3]	10.9 ± 1.2[3]	5.1 ± 0.2[3]	7.2 ± 0.4[2]

[a] Groups of 5-week old A/J female mice were fed AIN-76A diet for 1 week. Two h prior to sacrifice, corn oil, 5 μmol of PEITC, or 25 μmol of PEITC were given intragastrically to each mouse. Lung microsomes were made for the study.

[b] Values are the mean ± SD of four replications. Means with different superscripts in the same column are significantly ($P < 0.05$) different from each other determined by analysis of variance followed by the Newman-Keuls range test.

Table 5 *Role of preincubation and metabolism in the inactivation of microsomal NNK metabolism activity by PEITC*[a,b]

Preincubation time (min)	NADPH[c]	PEITC (nM)	(pmol/min/mg protein) HCHO	Keto alcohol	Keto acid	NNK-*N*-oxide	NNal
0		0	33.1 ± 2.5[1]	36.6 ± 3.1[1]	1.6 ± 0.1	23.4 ± 2.0[1]	12.1 ± 0.7[1]
0		25	21.5 ± 1.2[2]	23.2 ± 0.9[2]	ND[d]	18.2 ± 0.3[2]	9.2 ± 0.3[2]
0		50	12.1 ± 0.6[3]	12.8 ± 1.3[3]	ND	4.2 ± 0.2[3]	8.1 ± 0.8[2]
10	+	0	33.7 ± 1.9	36.8 ± 1.5	2.0 ± 0.2	20.8 ± 2.1	10.9 ± 0.5
10	−	0	31.6 ± 1.4	33.5 ± 2.7	1.6 ± 0.1	22.7 ± 1.8	10.1 ± 0.3
10	+	25	14.7 ± 1.2	14.2 ± 1.0	ND	7.2 ± 0.3	8.3 ± 0.7
10	−	25	16.2 ± 0.9	15.3 ± 1.4	ND	6.1 ± 0.3	7.5 ± 0.2
10	+	50	6.4 ± 0.7	7.1 ± 0.3	ND	1.4 ± 0.2	5.3 ± 0.2
10	−	50	6.9 ± 0.4	6.5 ± 0.5	ND	ND	8.4 ± 0.8
20	+	25	16.7 ± 1.1	16.9 ± 0.4	ND	9.6 ± 0.6	8.6 ± 1.8
20	−	25	17.1 ± 0.9	18.0 ± 1.2	ND	10.8 ± 1.3	9.6 ± 1.0

[a] Samples were preincubated for 0, 10, or 20 min. NNK metabolism was initiated by the addition of substrate and/or NADPH generating system. For the samples preincubated for 20 min, the reaction time was 20 min; for other samples it was 30 min.

[b] Values are the mean ± SD of three replications, except for the 0-min samples, which are the mean of six replications. Means with different superscripts in the same column are significantly ($P < 0.05$) different from each other as determined by analysis of variance followed by the Newman-Keuls range test. A Student's *t* test was performed on data comparing the effects of the preincubation in the presence and absence of NADPH-generating system. No significant difference was found.

[c] NADPH-generating system present (+) or absent (−).

[d] ND, not detectable.

formed via the α-hydroxylation of the methyl carbon of NNK. The nature of the α-hydroxylation of the methylene carbon, which leads to the formation of a methylating agent, however, was not elucidated in the present work. By studying O^6-methylguanine formation in the rat lung following NNK doses of 0.1 to 100 mg/kg/d, Belinsky *et al.* (25) suggested that there were low and high K_m pathways for the metabolism of NNK to a methylating agent. The present results provide further evidence for the presence of multiple K_m pathways in the activation of NNK. Since the carcinogenic dosage of NNK is rather low, the low K_m form of the enzyme should be more important in the activation of this carcinogen. Therefore, most of the present studies employed low NNK concentrations (10 μM).

Although results from studies with inhibitors should be interpreted with caution, the results in Table 2 suggest that cytochromes P-450 play a major role in the oxidative metabolism, but not in the reductive metabolism, of NNK. The results with methimazole suggest that the flavin-dependent monooxygenase is not important in the formation of keto alcohol but may be involved to a certain extent in the formation of keto acid and NNK-N-oxide. However, the possible nonspecific actions of the inhibitors made specific assignment difficult. The results with 9-hydroxyellipticine also suggest that enzymes related to cytochromes P-450IA1 and -2 are involved. However, because of the inter-species difference in the specificity and potency of the action of these inhibitors, additional studies are needed to substantiate this point.

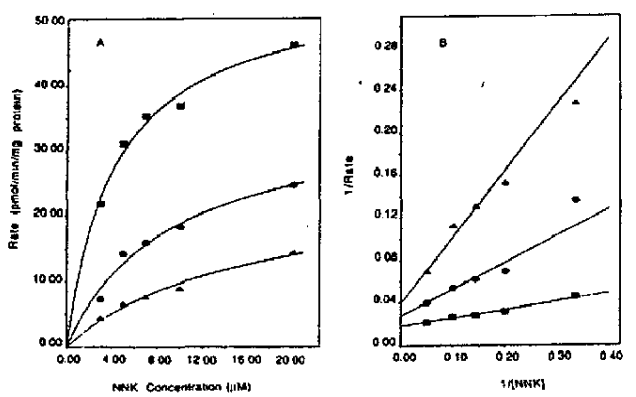

Fig. 4. Mechanisms of inhibition of keto alcohol formation by PEITC and PPITC. The incubation mixture consisted of a microsomal protein (0.1 mg); 10 μM NNK (1 μCi of [5-^{3}H]NNK and 1 μCi of [^{3}H-*methyl*]NNK); a NADPH-generating system; 100 mM sodium phosphate buffer, pH 7.4; 1 mM EDTA; 3 mM $MgCl_2$; and 50 nM PEITC (●), 50 nM PPITC (▲), or no inhibitor (■) in a total volume of 400 μl. Reactions were carried out for 30 min at 37°C. The activity is expressed as pmol keto alcohol/min/mg protein. *Points*, mean of four replications; difference between the replications was less than 10%. *A*, Rate versus [NNK] plot in the presence and absence of PEITC and PPITC. *B*, double-reciprocal plot showing the inhibition of keto alcohol formation by PEITC and PPITC.

The results obtained with antibodies (Table 3) suggested that, in mouse lung microsomes, enzymes immunochemically related to rat cytochromes P-450IIB1 and -2 can account for 25% of the activity for the formation of keto alcohol, whereas those to P-450IA1 may account for 15% of the activity. Orthologues of P-450IA2, if they exist in the mouse lung, did not play a role in NNK metabolism. However, the inter-species difference of immunoinhibition makes this interpretation less certain. The present results are different from those of Devereux *et al.* (10), who found that antibodies to rabbit cytochrome P-4502 (P-450IIB4, orthologous to rat P-450b) inhibited DNA methylation by rat lung microsomes 83%, suggesting a key role of cytochrome P-450b in the activation of NNK. Several factors might contribute to the differences: (*a*) the relative importance of P-450 in NNK metabolism may be different between rats and mice; (*b*) Devereux *et al.* (10) studied the product of methylene oxidation, whereas we dealt with the products of the methyl oxidation; and (*c*) Devereux *et al.* (10) used 2 mM NNK, whereas we used 10 μM NNK as the substrate. It is possible that cytochrome P-450IIB1 is the major isozyme involved in the high K_m pathway for NNK activation but not in the low K_m pathway. The metabolism of NNK by purified rat liver P-450IIB1 in a reconstituted system displayed an apparent K_m of 0.46 mM.[5] However, the K_m in a reconstituted system may be higher than the K_m displayed by the same enzyme in microsomes.

The inhibitory action of PEITC is consistent with its inhibition of NNK-induced lung DNA methylation and carcinogenesis (13, 26). Two types of inhibition were demonstrated in the present work: (*a*) competitive inhibition due to competition between PEITC and NNK for the active site of the enzyme (most likely cytochrome P-450); and (*b*) a noncompetitive component of the inhibition possibly due to the chemical inactivation of cytochrome P-450 by PEITC. Both mechanisms should also be applicable *in vivo*; the relative importance depends on the time and dose of the exposure to PEITC and

[5] Z. Y. Guo and C. S. Yang, unpublished results.

Table 6 *Percent inhibition of NNK metabolism by isothiocyanates*[a,b]

Isothiocyanate concentration	HCHO	Keto alcohol	Keto acid	NNK-*N*-oxide	NNal
PITC					
25 nM	8 ± 2	5 ± 1	21 ± 6	0	2 ± 1
100 nM		7 ± 6	19 ± 3	42 ± 5	0
BITC					
25 nM	16 ± 5	12 ± 8	24 ± 9	2 ± 2	0
100 nM		70 ± 9	100	81 ± 6	0
PEITC					
25 nM	28 ± 4	22 ± 3	29 ± 5	19 ± 4	4 ± 3
100 nM		65 ± 5	100	80 ± 3	4 ± 1
PPITC					
25 nM	37 ± 6	29 ± 9	49 ± 5	24 ± 8	2 ± 2
100 nM		92 ± 2	100	100	0
PBITC					
25 nM	49 ± 2	46 ± 1	73 ± 4	56 ± 7	0
100 nM		96 ± 3	100	93 ± 6	0

[a] NNK metabolism in control lung microsomes was 32.1, 34.8, 2.4, 25.3, and 11.5 pmol/min/mg protein for HCHO, keto alcohol, keto acid, NNK-*N*-oxide, and NNal, respectively.

[b] Values are the mean ± SD of four replications.

NNK. The lower K_i values displayed by PPITC than PEITC are consistent with the results (Table 6) that the former was a more potent inhibitor than the latter. The potency and relative ranking in the inhibitory action of isothiocyanates observed herein are also consistent with the inhibitory action of these compounds in carcinogenesis experiments (15). When PEITC was given to mice 2 h before a dose of NNK, it effectively inhibited lung carcinogenesis (13). This observation suggests that inhibition of NNK metabolism is the most likely mechanism for the inhibition of carcinogenesis. The present results sheds light on the enzymes and mechanisms involved in the inhibition of NNK metabolism by isothiocyanates.

Exposure to NNK occurs by way of use of tobacco products. It has been estimated that the daily exposure to NNK through cigarette smoking for two-pack-a-day smokers is about 8 μg/person (25). Chronic exposure to low doses of NNK can result in an increased incidence of malignant lung tumors, as has been found in animal studies (4, 27). The ability to effectively inhibit the carcinogenicity of NNK by isothiocyanates demonstrates the potential usefulness of these compounds in cancer prevention. The present finding that PEITC can inhibit NNK metabolism competitively suggests that low levels of PEITC obtained from the human diet may be useful for the inhibition of low-level exposure to NNK.

ACKNOWLEDGMENTS

The authors would like to thank Mao-Jung Lee for expert technical assistance, Fang Xiao for the pentoxyresorufin dealkylase analysis; Drs. John Brady, Theodore Chase, Jr., and Jun-Yan Hong for helpful discussions and suggestions; and Dorothy Wong for excellent secretarial assistance in the preparation of this manuscript.

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